Novel Recombinant Anticoagulant Proteins

This application claims priority from provisional U.S. Application No. 60/386932, filed June 6, 2002, the disclosure of which is herein incorporated by reference in its entirety.

Background of the Invention

The present invention relates in general to anticoagulant proteins, and in particular to novel recombinant blood coagulation inhibitors.

Tissue factor (TF) is generally considered to be the physiological trigger of the blood coagulation in normal hemostasis and in a variety of coagulopathic and thrombotic diseases. TF is an integral membrane protein that is normally present on the surface of certain extra-vascular cell types, but can also be induced to express on endothelium and monocytes upon stimulation [reviewed in (1)]. Based on studies in whole blood and re-constituted plasma systems (2-5), the key events of TF-initiated blood clotting can be schematically illustrated as in Fig. 1. Upon exposure, TF forms a complex with factor VII/VIIa present in the circulating blood. The resulting extrinsic tenase complex (TF/VIIa) initiates the clotting cascade by activating small amounts of factors IX and X on the TF-bearing cells/microparticles. The TF/VIIa-activated factors IXa and Xa play distinct roles in the subsequent coagulation reactions. In a complex with factor Va/V on TF-bearing membrane surface, factor Xa generates a small amount of thrombin that partially activates platelets, cleaves fibrinogen to form an initial clot, and converts factors V, VIII, and XI to their active forms. Subsequent to this initiation phase, propagation of thrombin generation begins. During the propagation phase, activated platelets provide an anionic membrane surface for the assembly of intrinsic tenase (VIIIa/IXa) and prothrombinase (Va/Xa) complexes, which very efficiently activate factor X and prothrombin, respectively, leading to explosive thrombin generation and consolidation of the fibrin-platelet plug. Three plasma anticoagulant systems regulate the clotting cascade, each acting at a different point in the cascade. Tissue factor pathway inhibitor (TFPI) influences the initiation phase by forming a TFPI-Xa inhibitory complex that inhibits TF/VIIa through feedback inhibition; antithrombin III (AT III) primarily exerts its effect by inhibiting free thrombin and Xa in the propagation phase; and activated protein C (APC) affects the duration of the propagation by proteolytically inactivating Va and VIIIa.

The availability of anionic phospholipid, chiefly phosphatidyl-L-serine (PS), is important for the assembly and expression of catalytic activities of the membrane-associated coagulation enzyme complexes (extrinsic tenase, intrinsic tenase, prothrombinase and XIa) that drive the initiation and propagation of the coagulation cascade. Plasma membrane phospholipids of mammalian cells are normally asymmetrically distributed, with PS being exclusively sequestered in the inner membrane leaflet (6). Because of PS sequestration, intact quiescent cells are normally not procoagulant. In circumstances of cell activation, cell injury, or in response to apoptotic stimuli, phospholipid asymmetry across the plasma membrane collapses, resulting in exposure of PS on the membrane surface and shedding of membrane "microparticles". The exposure of PS allows assembly of enzyme/cofactor complexes and interaction with their substrates on the membrane surface, thereby enhancing the efficiency of the coagulation reactions (7-9). TF/VII(a) complex formed on intact cells is often cryptic in enzymatic activity towards its substrates. A many fold increase in TF/VIIa activity (de-encryption) is observed when PS becomes available on the membrane surface after cell disruption, treatments with various agents, or induction of apoptosis (10-14). The rate of factor X activation by TF reconstituted with vesicles composed of phosphatidylcholine (PC) alone is less than 5 % of that observed with PS-PC vesicles (15). These observations suggest that concomitant expression of TF and exposure of PS on the membrane surface are important in the initiation of coagulation. In the processes of hemostasis/thrombosis, platelets are known to provide an anionic membrane surface for the assembly of intrinsic tenase (VIIIa/IXa) and prothrombinase (Va/Xa) (7,16). Upon platelet activation, PS rapidly appears on the platelet membrane surface. Interaction of factor VIIIa with the anionic lipid creates a Ca^{++}-dependent high-affinity binding site for factor IXa, leading to the formation of the intrinsic tenase complex. Likewise, binding of factor Va to anionic lipid promotes Ca^{++}-dependent binding of factor Xa, forming the prothrombinase complex. Factor XIa also depends on the PS-exposed membrane for efficient catalysis of the conversion of factor IX into factor IXa.

TFPI is a multivalent Kunitz-type inhibitor that regulates the initiation of the tissue factor pathway of coagulation in the human vascular system (17). TFPI inhibits factor Xa directly, and in a factor Xa-dependent manner, produces a feedback inhibition of TF/VIIa complex and thus dampens the protease cascade of the tissue factor pathway. Although TFPI is physiologically very important in the regulation of tissue factor pathway, its

development for clinical antithrombotic therapy is currently limited by the large doses required for it to effectively interrupt vascular thrombosis (18-20).

Several other, naturally occurring Kunitz-type inhibitors that bind factors VIIa, IXa, Xa, and XIa of the tissue factor pathway have also been described. These include leech-derived Antistasin (ATS) (21), Tick Anticoagulant Peptide (TAP) (22), and two Ancylostoma caninum Anticoagulant Peptides (AcAP5 and AcAP6) (23) that inhibit factor Xa specifically; another Ancylostoma caninum Anticoagulant Peptide (AcAPc2) that inhibits VIIa (23); and a Kunitz-inhibitory domain of amyloid β-protein precursor (K_{APP}) that inhibits factors VIIa, IXa, Xa, and XIa (24-27). Using site-specific mutagenesis and phage display technology, two series of K_{APP} and aprotinin (bovine pancreatic trypsin inhibitor) homologs with very high affinity (sub-nanomolar K_i) toward different coagulation proteases (TF/VIIa, Xa, XIa, and Kallikrein etc.) have been created (28-31). However, the anticoagulant potencies of these mutants are quite low in *in vitro* coagulation assays (tissue factor-initiated clotting and activated partial thromboplastin time). The aprotinin homologs also require very high doses to achieve antithrombotic effect in an *in vivo* vascular trauma model (31).

Brief Summary of the Invention

In accordance with the present invention, novel recombinant anticoagulant proteins, methods and materials relating to their production, and methods of their use in treatment are provided.

A novel series of recombinant anticoagulant fusion proteins are preferably created by linking annexin V (ANV) (SEQ ID NO: 10), a phosphatidylserine (PS) binding protein, to Kunitz-type protease inhibitors (KPI) targeting the serine proteases in the enzymatic complexes. The resulting fusion proteins exhibit much stronger anticoagulant activities than their component proteins. Several of these constructs possess far greater potencies than TFPI, the natural inhibitor of TF-initiated coagulation in blood. The annexinV:Kunitz-type inhibitor (ANV:KPI) fusions represent a new class of anticoagulants that specifically target the coagulation enzyme complexes on the procoagulant PS-exposed membrane surface, and are useful as anti-thrombotic therapeutic agents with an ability to passivate thrombogenic vessel wall and associated thrombi.

Therefore, in one embodiment there are provided recombinant anticoagulant proteins, each comprising a fusion of annexin V (ANV) (SEQ ID NO: 10) and a Kunitz protease inhibitor (KPI). Alternative embodiments of the recombinant anticoagulant protein include, for example, fusion of ANV with Tick Anticoagulant Peptide (TAP) (SEQ ID NO: 1), with a variant of aprotinin (6L15) (SEQ ID NO: 2), with the Kunitz-inhibitory domain of amyloid β-protein precursor (K_{APP}) (SEQ ID NO: 3), and with $KK_{TFPI\ 22\text{-}160}$ (SEQ ID NO: 4).

In another embodiment there is provided an antithrombotic composition comprising a recombinant anticoagulant protein comprising a fusion of annexin V (ANV) (SEQ ID NO: 10) and a Kunitz protease inhibitor (KPI). Alternative embodiments of the antithrombotic composition include, for example, TAP-ANV (SEQ ID NO: 1), ANV-6L15 (SEQ ID NO: 2), ANV-K_{APP} (SEQ ID NO: 3), and ANV-$KK_{TFPI\ 22\text{-}160}$ (SEQ ID NO: 4).

In another embodiment there is provided a method of inhibiting blood coagulation in a mammalian subject comprising administering to the subject an effective amount of a recombinant anticoagulant protein comprising a fusion of annexin V (ANV) (SEQ ID NO: 10) and a Kunitz protease inhibitor (KPI).

In another embodiment there is provided a method of producing a recombinant anticoagulant protein comprising linking annexin V (ANV) (SEQ ID NO: 10) and a Kunitz protease inhibitor (KPI).

In another embodiment there is provided a method of treating or preventing an excess of thrombotic activity in a subject in need of such treatment or prevention, said method comprising administering to the subject an effective amount of an antithrombotic composition comprising a fusion of annexin V (ANV) (SEQ. ID NO: 10) and a Kunitz protease inhibitor (KPI).

In another embodiment there is provided a recombinant DNA molecule comprising a first DNA sequence encoding annexin V (ANV) (SEQ ID NO: 9) and second DNA sequence encoding a Kunitz protease inhibitor (KPI). Alternative embodiments of the recombinant DNA molecule include, for example, DNA sequences selected from the group consisting of TAP-ANV (SEQ ID NO: 5), ANV-6L15 (SEQ ID NO: 6), ANV-K_{APP} (SEQ ID NO: 7), and ANV-KK_{TFPI} (SEQ ID NO: 8), or conservatively substituted variants thereof.

In another embodiment there is provided a process for the preparation of a cell line expressing a recombinant anticoagulant protein comprising a fusion of annexin V (ANV) (SEQ ID NO: 10) and a Kunitz protease inhibitor (KPI), the process comprising stably transfecting a host cell with a recombinant expression vector comprising a cDNA sequence encoding ANV or conservatively substituted variants thereof, and a cDNA sequence encoding a KPI.

In another embodiment there is provided a recombinant expression vector comprising a first nucleotide sequence encoding annexin V (ANV) (SEQ ID NO: 9), Cys^{315}-to-Ala mutation of ANV (SEQ ID NO: 14), or conservatively substituted variants thereof, and a second nucleotide sequence of a Kunitz protease inhibitor (KPI) together with additional sequences capable of directing the synthesis of a recombinant anticoagulant protein comprising a fusion of ANV and a KPI, in a culture of stably tranfected cells. Alternative embodiments of the recombinant expression vector include, for example, a nucleotide sequence selected from the group of TAP-ANV (SEQ ID NO: 5), ANV-6L15 (SEQ ID NO: 6), ANV-K_{APP} (SEQ ID NO: 7), and ANV-KK_{TFPI} (SEQ ID NO: 8), or conservatively substituted variants thereof.

Brief Description of the Drawings

Fig. 1. Schematic of TF-initiated clotting.

Factor VII binds to TF and is activated to VIIa on the TF-bearing cells/microparticles. The TF/VIIa complex activates both factor IX and factor X. The factor Xa generates a small amount of thrombin (IIa) locally. This small amount of thrombin activates platelets, activates factor V, releases factor VIII from von Willebrand factor and activates it, and activates factor XI. TF/VIIa-activated IXa can then bind to the VIIIa on the activated platelet to form an intrinsic tenase (VIIIa/IXa) that activates factor X efficiently. The platelet-generated Xa binds Va to form a prothrombinase (Va/Xa) that promotes large-scale conversion of prothrombin (II) to thrombin. Three plasma anticoagulant systems regulate the coagulation cascade: TFPI directly inhibits Xa, and in a Xa-dependent manner produces a feedback inhibition of TF/VIIa; AT III mainly inhibits Xa and thrombin; and APC proteolytically inactivates Va and VIIIa. Adapted from Roberts et al. (5) and Mann K et al. (3).

Fig. 2. Schematic of annexin V and its fusion products with various Kunitz-type inhibitors.

ANV, annexin V (SEQ ID NO: 10); TAP-ANV (SEQ ID NO: 1), ala-tick anticoagulant peptide linked to annexin V by Gly-Ser dipeptide; ANV-6L15 (SEQ ID NO: 2), annexin V linked to 6L15 (a Kunitz inhibitor with high affinity for TF/VIIa); ANV-K_{APP} (SEQ ID NO: 3), annexin V linked to K_{APP} (Kunitz inhibitory domain of amyloid β-protein precursor); ANV-KK_{TFPI} (SEQ ID NO: 4), annexin V linked to KK_{TFPI} ($TFPI_{22-161}$ containing Kunitz-1 and Kunitz-2 domains).

Fig. 3. SDS-PAGE analysis of purified ANV and its fusion products with various Kunitz-type protease inhibitors.

Samples were analyzed by 12% SDS-PAGE under non-reducing (A) or reducing (B) conditions followed by Coomassie blue staining. All samples were boiled for 3min without (A), or with (B) 50 mM dithiothreotol. Approximately 5 μg proteins were loaded on each lane. Lane 1, molecular weight marker; lane 2, ANV-KK_{TFPI} (SEQ ID NO: 4); lane 3, ANV-6L15 (SEQ ID NO: 2); lane 4, TAP-ANV (SEQ ID NO: 1); lane 5 ANV-K_{APP} (SEQ ID NO: 3); lane 6, ANV (SEQ ID NO: 10).

Fig. 4. Inhibition of porcine trypsin and bovine factor Xa by various purified inhibitors.

Inhibitions of trypsin and bovine factor X were measured by amidolytic assays as described in Methods herein below. The concentrations of active trypsin and bovine factor Xa were determined by active site titration with 4-nitrophenyl *p'*-guanidinobenzoate (41,42). The concentrations of purified inhibitors were determined by absorbance measurement at 280nm using molar extinction coefficients of 28170, 7120, 39550, 18500, 31300, and 30170 for ANV-6L15, 6L15, TAP-ANV, TAP, ANV-K_{APP}, and ANV-KK_{TFPI}, respectively. (A) Inhibition of trypsin by ANV-6L15; (B) Inhibition of trypsin by 6L15; (C) Inhibition of factor Xa by TAP-ANV; (D) Inhibition of factor Xa by TAP; (E) Inhibition of trypsin by ANV-K_{APP}; (F) Inhibition of factor Xa by ANV-KK_{TFPI}.

Fig. 5. Effects of various inhibitors in activated partial thromboplastin time (APTT) assay.

APTT assay was carried out using an ACL 200 Coagulometer and APTT-SP reagent (Instrument Laboratories). Pooled human plasma (180 μl) was mixed with 20μl of various inhibitors to attain the indicated final concentrations for the assay. The plasma with control buffer had a clotting time of 40.7 sec. TFPI (m) refers to mammalian C127 cell-derived FL-TFPI.

Detailed Description of the Invention

The following detailed description of the invention taken in conjunction with the accompanying drawings is provided to further illustrate the invention and preferred embodiments in greater detail.

The blood coagulation cascade proceeds primarily via the formation of coagulation enzyme complexes, each consisting of a serine protease associated with a membrane-bound cofactor/recptor on an anionic membrane surface. These complexes are conventionally named extrinsic tenase (factor VIIa-tissue factor), intrinsic tenase (factor IXa-factor VIIIa), prothrombinase (factor Xa-factor Va) and XIa complex.

As described herein, a novel series of recombinant anticoagulant fusion proteins are created, for example, by linking annexin V (ANV) (SEQ ID NO: 10), a phosphatidylserine (PS) binding protein, to a Kunitz-type protease inhibitor (KPI) targeting the serine proteases in the enzymatic complexes. The resulting fusion proteins exhibit much stronger anticoagulant activities than their component proteins alone or even additively. For convenience, these recombinant anticoagulant fusion proteins are abbreviated ANV:KPI. These fusion proteins utilize the high affinity of ANV for phosphatidyl-L-serine (PS) (32) and various KPI's for inhibition of the serine proteases in membrane-associated coagulation complexes in the blood coagulation cascade. Several of these novel constructs possess far greater potencies than TFPI, the natural inhibitor of TF-initiated coagulation in blood. The annexinV:Kunitz-type protease inhibitor (ANV:KPI) fusion proteins represent a new class of anticoagulants that specifically target the coagulation enzyme complexes on the procoagulant PS-exposed membrane surface, and are useful as anti-thrombotic therapeutic agents with an ability to passivate thrombogenic vessel wall and associated thrombi. The novel fusion proteins will be useful in the treatment of diseases and

conditions involving an excess of thrombogenesis, including arterial thrombotic events such as unstable angina, myocardial infarction, sudden cardiac death, ischemic stroke, ruptured aneurisms, intermittent claudication, and critical limb ischemia; venous thrombosis such as deep venous thrombosis, pulmonary embolism, thrombophlebitis, and chronic venous insufficiency; and other clinical conditions such as surgical thrombosis, prosthetic heart valve, atherosclerosis, restenosis, ischemia reperfusion injury, sepsis, disseminated intravascular coagulation, acute lung injury, malignancy, chronic renal failure, nephrotic syndrome, crescentic glomerulonephritis, diabetes, sickle cell anemia, thalassemia, antiphospholipid syndrome, extra-corporeal circulation, hemodialysis, peritoneal dialysis and annexinopathies.

In order to further illustrate the invention, the following specific laboratory examples were carried out although it will be understood that the invention is not limited to these specific examples or the details described therein.

EXAMPLES

Materials and Methods

Reagents

Urea (sequenal grade) and Brij 35 were obtained from Pierce. Mixed bed resin AG501-X8, SDS-PAGE reagents, and molecular weight markers were purchased from BioRad. Dade Innovin® was from Baxter Diagnostics Inc. (Deerfield, IL). APTT-SP was from Instrumentation Laboratory (Lexington, MA). Bovine factor Xa was supplied by American Diagnostica, Inc. (Greenwich, CT). Trypsin, *p*-nitrophenyl *p'*-guanidinobenzoate HCl, bovine brain extract, cholesterol, and diacetylphosphate were from Sigma (St. Louis, MO). The synthetic substrates, S2444 and S2765 were obtained from diaPharma (West Chester, OH). Freshly frozen human plasma was purchased from Taipei Blood Center. Mammalian C127 cell- and *E. coli*- derived recombinant TFPIs were prepared as described before (33,34). Recombinant X-K1 (C-terminal peptide of human factor X fused with the first Kunitz domain of TFPI) (35), and TFPI1-160 were gifts of Dr. George Broze, Jr., (Washington University). Yeast-derived recombinant TAP was a gift from Dr. Dana Abendschein (Washington University).

Cloning of cDNA for annexin V

ANV cDNA, lacking an initiation Met codon and a stop codon, was generated from human placental mRNA by PCR using ANV reverse primer 1 (5'-ATCAAGCTTATGCAT GTCATCTTCTCCACAGAG-3') (SEQ ID NO: 11) and forward primer 2 (5'-GATCGGAT CCAGTCTGGTCCTGCTTCACCTT -3') (SEQ ID NO: 12). ATGCAT is the site of restriction enzyme *Nsi* I used for ligating the 6L15, K_{APP}, or $KK_{TFPI22-161}$ gene fragment. ANV cDNA mutation of Cys^{315}-to-Ala was created by PCR using oligonucleotide X (5'-CG TGACATGCATGTCATCTTCTCC**AGC**GAGCA-3') (SEQ ID NO: 13), in which the bolded GC was changed from CA in order to replace the original codon of Cys into Ala. Recombinant ANV was expressed without mutation of Cys^{315}. For all other ANV: KPI fusions, ANV cDNA with Cys^{315}-to-Ala mutation (SEQ ID NO: 14) was used. The position of Cys/Ala was numbered as 315 in the PCR-amplified ANV cDNA lacking an initiation Met codon.

Construction of 6L15, TAP, and K_{APP} genes

The synthetic gene encoding 6L15 was constructed from 3 pairs of overlapping oligonucleotides. The three forward oligomers are: BP-1 (5'-TCCGGACTTCTGCCT GGAACCGCCGTACGACGGTCCGTGCCGTGCTCTGCACCTGCGTTACTTC-3') (SEQ ID NO: 15); BP-2 (5'-TACAATGCAAAGGCAGGCCTGTGTCAGACCTTCTA CTACGGCGGTTG CCTGGCTAAGCGT-3') (SEQ ID NO: 16); and BP-3 (5'-AACAA CTTCGAATCCGCGGAACACTGCATGCGTACTTGCGGTGGTGCTTA-3') (SEQ ID NO: 17). The three reverse oligomers are: BP-1-3'(5'-ACGCAGGTGCAGAGCACGGCA CGGACCGTCGTACGGCGGTTCCAGGCAGAAGTCCGGATGCAT-3') (SEQ ID NO: 18); BP-2-3' (5'-AGCCAGGCAACCGCCGTAGTAGAA GGTCTGACACAGGCCTGCC TTTGCATTGTAGAAGTA-3') (SEQ ID NO: 19); ; and BP-3-3' (5'-AGCTTAAGCAC CACCGCAAGTACGCATGCAGTCTTCCGCGGATTCGAAGTTGTTACGCTT-3') (SEQ ID NO: 20); . The internal oligomers were phosphorylated with T_4-polynucleotide kinase. The three complementary oligonucleotide pairs were annealed separately by heating to 95^0 and slow cooling to room temperature. The annealed oligonucleotide pairs were then ligated with T_4-DNA ligase to form 6L15 gene. The *Nsi*I restriction enzyme site ATGCAT was designed in the oligomer sequence of BP-1-3' by changing original codon of Arg^1 into His for the ligating to the ANV gene fragment. For expression of 6L15, the

original codon of Arg^1 was replaced by Ala. The synthetic 6L15 gene consists of the following sequence:

GCT CCG GAC TTC TGC CTG GAA CCG CCG TAC GAC GGT CCG TGC CGT GCT CTG CAC CTG CGT TAC TTC TAC AAT GCA AAG GCA GGC CTG TGT CAG ACC TTC TAC TAC GGC GGT TGC CTG GCT AAG CGT AAC AAC TTC GAA TCC GCG GAA GAC TGC ATG CGT ACT TGC GGT GGT GCT TAA (SEQ ID NO: 21).

The synthetic ala-TAP gene was synthesized from synthetic oligonucleotides according to Neepert et al. (36). The synthetic ala-TAP gene consists of the following sequence:

GCT TAC AAC CGT CTG TGC ATC AAA CCG CGT GAC TGG ATC GAC GAA TGC GAC TCC AAC GAA GGT GGT GAA CGT GCT TAC TTC CGT AAC GGT AAA GGT GGT TGC GAC TCC TTC TGG ATC TGC CCG GAA GAC CAC ACC GGT GCT GAC TAC TAC TCC TCC TAC CGT GAC TGC TTC AAC GCT TGC ATC TAA (SEQ ID NO: 22);

The synthetic K_{APP} gene with flanking sequences was constructed from two pairs of overlapping synthetic oligonucleotides. The two forward oligomers are: K_{APP}-1 (5'-GGCC CTACCCCACAGATACGGAGTTGCCACCACTGAAACTTGAGGTTGTTAGAGAGG TTTGTTCTGAGCAAGCTGAGACTGGTCCATGTAGAGCTATGATTTCTAGATGGT ACTTCGACGTT-3') (SEQ ID NO: 23); , and K_{APP}-2 (5'-ACTGAGGGTAAGTGTGCT CCATTCTTCTACGGTGGTTGTGGTGGTAACAGAAACAACTTCGACACTGAGGA GTACTGTATGGCTGTTTGTGGTTCTGCTATTTAAATGCATTGATGA-3') (SEQ ID NO: 24). The two reverse oligomers are: K_{APP}-1-3'(5'-CTCAGTAACGTCGAAGTACCA TCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGCTCAGAACAAACCTC TCTAACAACCTCAAGTTTCAGTGGTGGCAACTCCGTATCTGTGGGGTAG-3') (SEQ ID NO: 25); and K_{APP}-2-3'(5'-AGCTTCATCAATGCATTTAAATAGCAGAACC ACAAACAGCCATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACA ACCACCGTAGAAGAATGGAGCACACTTACC-3') (SEQ ID NO: 26). The underlined are complementary sequences coding for the K_{APP} domain. The oligomers were phosphorylated with T_4-polynucleotide kinase.and annealed by heating to 95^0 and slow cooling to room temperature. The annealed oligonucleotide pairs were then ligated with T_4-DNA ligase. The K_{APP} domain of the synthetic gene consists of the following sequence:

GAG GTT TGT TCT GAG CAA GCT GAG ACT GGT CCA TGT AGA GCT ATG ATT

TCT AGA TGG TAC TTC GAC GTT ACT GAG GGT AAG TGT GCT CCA TTC TTC TAC GGT GGT TGT GGT GGT AAC AGA AAC AAC TTC GAC ACT GAG GAG TAC TGT ATG GCT GTT TGT GGT TCT GCT ATT TAA (SEQ ID NO: 27)

Construction of E. coli expression plasmids

To construct the plasmids for expression of ANV-6L15 and ANV-KK_{TFPI}, the following primers were used for PCR amplification and subcloning into pET20b expression vector: ANV-nde (5'- G GAATTCCATATGGCACAGGTTCTCAGAGG-3') (SEQ ID NO: 28), ANV-nsi (5'-CCAATGCATGTCATCTTCTCCAGC-3')) (SEQ ID NO: 29),

6L15-nsi (5'-CCAATGCATCCGGACTTCTGCCTG-3')) (SEQ ID NO: 30),

KK_{TFPI}-nsi (5'-CCAATGCATTCATTTTGTGCATTC-3')) (SEQ ID NO: 31),

6L15-sal (5'-ACGCGTCGACTTA AGCACCACCGCAAG -3') (SEQ ID NO: 32), and

KK_{TFPI}-sal (5'- ACGCGTCGACTTAGGTTCCATA ATTATCC -3') (SEQ ID NO: 33).

The sequence underlined is a *Nde* I restriction enzyme site and boxed is the cutting site for *Sal* I. The underlined ATGCAT is Nsi I restriction enzyme site used for gene fusion. The enlarged ATG is the initiation codon of Met and the TTA is a complimentary sequence to the stop codon of TAA. The $KK_{TFPI22\text{-}161}$ gene fragment was obtained by PCR amplification from a full-length TFPI cDNA clone (34) using primers KK_{TFPI}-nsi (SEQ ID NO: 31) and KK_{TFPI}-sal (SEQ ID NO: 33). The PCR amplified gene fragment of ANV was digested with *Nde*I and *Nsi*I restriction enzymes and linked to *Nsi*I and *Sal*I digested 6L15 (or KK_{TFPI}) PCR fragment. The fusion gene was ligated into the expression vector of pET20b(+) which was linearized with *Nde*I and *Sal* I restriction enzymes.

To construct the plasmid for expression of TAP-ANV, the following primers were used for gene fusion and subcloning into pET20b:

TAP-nde (5'- GGAATTCCATATGGCTTACAACCGTCTGTG -3') (SEQ ID NO: 34);

TAP-bam (5'- CGGGATCCGATGCAAGCGTTGAAGCAG -3') (SEQ ID NO: 35);

ANV-bam (5'- CGGGATCCGCACAGGTTCTCAGAGGC -3') (SEQ ID NO: 36);

ANV-sal (5'- ACGCGTCGACTTAGTCATCTTCTCCAGCG -3') (SEQ ID NO: 37).

The PCR amplified gene fragment of TAP was digested with *Nde*I and *Bam*HI restriction enzymes and linked to *Bam*HI and *Sal*I digested ANV gene fragment. The fusion gene was inserted into the expression vector of pET20b(+) which was also linearized with *Nde*I and *Sal* I restriction enzymes.

The desired recombinant plasmids were screened by PCR and DNA sequence determination. The expression plasmids are designated pET20b-AB8, pET20b-AKK11, and pET20b-TAP-A, which expressed intracellularly the recombinant proteins of ANV-6L15 and ANV-KK_{TFPI}, and TAP-ANV, respectively, in *E. coli* under the control of T7 promoter.

To express ANV and 6L15 for the purpose of comparison, the PCR-generated gene fragments of ANV and 6L15, respectively, were inserted into the plasmid using the same strategy for *E. coli* expression.

E. coli expression

E. coli BL21 (DE3) pLysS [(F^- *ompT* *hsd*S_B (r_B^-,m_B^-) *gal dcm* (DE3) pLysS ($Cam)^R$)] (Novagene, Madison, WI) was used for expression of recombinant proteins. *E. coli* DH5α [(F^- (ϕ80d *lacZ*ΔM15) Δ(*lacZYA-argF*)U169 *endA1* *recA1* *hsd*R17(r_K- m_K+) *deoR* *thi*-1 *sup*E44 *gyr*A96 *rel*A1 λ^-)] was used for construction of expression plasmids. The expression plasmid was propagated and isolated from *E. coli* DH5α and was transformed into the frozen competent cells of *E. coli* BL21. A single colony was inoculated into a 25-ml LB broth (containing 100 mg/L ampicillin and 34 mg/L chloroamphenicol), and grown overnight at 37^0 with vigorous shaking. Ten ml of the overnight culture was inoculated into 1 liter of the same medium in a 2.8L flask (Nalgene) and maintained at 37^0 until the OD_{600} of the culture reached 0.5. The culture was induced by adding IPTG (Promega) to a final concentration of 1 mM and continuously shaking at 37^0 for 4 hours. The *E. coli* cells were harvested by centrifugation at 7000 rpm for 12 min. The cell pellet was frozen at – 80^0 for further use.

Construction of Yeast expression plasmid

The *Pichia* expression vector of pPIC9, utilizes the strong and highly inducible P_{AOX1} promoter and α-factor signal peptide for high level expression and secretion of target

proteins. The fragment containing the gene of interest was cloned in frame with the secretion signal peptide flanked by *Xho* I and *Not* I sites, the sequences from the *Xho* I site to the initiation codon of the target gene encoding the protease site of KEX2 must be created for occurrence of efficient cleavage of the fusion protein. The primers designed for generating PCR fragment of interest for cloning into vector pPIC9 were ANV-xho (5'-CCG *CTCGAG* AAA AGA GCA CAG GTT CTC AGA G-3') (SEQ ID NO: 38), K_{APP}-not (5'-ATA AGA AT **GCGGCCGC** TTA AAT AGC AGA ACC AC-3') (SEQ ID NO: 39), ANV-ecov (5'-CGC GAT ATC ATC TTC TCC AGC GAG-3') (SEQ ID NO: 40), 5'-K_{APP} (5'-GAG GTT TGT TCT GAG CAA GC-3') (SEQ ID NO: 41). The sequences *CTCGAG* and **GCGGCCGC** are *Xho* I and *Not* I restriction enzyme sites, respectively, used for editing the gene fragment and ligating into the vector pPIC9. The *CTC GAG* AAA AGA encoded 4 amino acids, Leu-Glu-Lys-Arg, which is a typical cleavage site for KEX 2 protease, so the following codon in the primer was designed to be the first codon (shown in enlarged text) of the secreted protein of interest. For generating the ANV-K_{APP} fusion gene, we designed primer ANV-ecov which would create *Eco*RV site (GATATC) located at 3'-end of the ANV gene fragment without changing the last encoded amino acid (Asp). 5'-K_{APP} primer is a forward sequence of K_{APP} gene from the initiation codon of Glu (GAG). The K_{APP} gene fragment amplified by primers 5'-K_{APP} and K_{APP}-not was blunt-end ligated to ANV gene amplified by primers ANV-xho and ANV-ecov and digested by *Eco*RV to generate the fusion gene of ANV-K_{APP}. The fusion gene was digested by *Xho* I and *Not* I restriction enzymes and ligated to the pPIC9, which was linearized using the same enzymes. The ligation mixture was transformed into *E. coli* DH5α and the desired clone was screened by PCR and confirmed by DNA sequence analysis to identify the in frame amino acid sequence along with α-factor signal peptide. The resulting plasmid was pPIC9 ANV-K_{APP}.

Pichia expression

The yeast expression plasmid was propagated and isolated from *E. coli* DH5α. Integration was targeted by digesting the expression plasmid with *Sac* I restriction enzyme prior to transformation. The α-factor fused gene cassette including *His*4 as the selection marker was inserted into the genome of *P. pastoris* GS115 (*his*4) at the *AOX*1 locus via electroporation (37). The recombinant strains were selected by growing from the MD (minimal dextrose medium, 1.34 % yeast nitrogen base without amino acid-$4x10^{-5}$ %

biotin-2 % dextrose-1.5 % bacto-agar) plate through the *His*4 compensation.

A single colony of *P. pastoris* GS115 recombinant strain from the MD plate was inoculated into 2 ml of BMGY medium (buffered glycerol complex medium, 1 % yeast extract-2 % peptone-100 mM potassium phosphate, pH 6.0-1.34 % yeast nitrogen base without amino acid-$4x10^{-5}$ % biotin-1 % glycerol) in 10 cm long Pyrex tube and grown at 30^0 with vigorous shaking at 200 rpm overnight until the OD_{600} of the culture reached 2-6. One ml of culture was harvested by centrifugation and resuspended into 3 ml of BMMY medium (buffered methanol complex medium, 1 % yeast extract-2 % peptone-100 mM potassium phosphate, pH 6.0-1.34 % yeast nitrogen base without amino acid-$4x10^{-5}$ % biotin-0.5 % methanol) in 15 cm long Pyrex tube. The culture was maintained at 30^0 with vigorous shaking at 200 rpm for 24 hours for expression of the secreted protein. The cells were concentrated by centrifugation at 12,000 rpm for 10 minutes and the supernatant was assayed for inhibitory activity against trypsin. Ten µl of the supernatant was subjected to 12 % SDS-PAGE and the expressed ANV-K_{app} was detected by Western blot.

For large-Scale Expression of ANV-K_{APP} in *Pichia*, a single colony of *P. pastoris* GS115 recombinant strain from the fresh MD plate was inoculated into 25 ml BMGY medium in a 300-ml flask and was grown at 30^0 with vigorous shaking at 200 rpm for 2 days. This late log phase culture was used to inoculate 400 ml fresh BMGY medium to a final OD_{600} of 0.1 in 1 L flask. The culture was maintained at 30^0 until OD_{600} reached 2. The cells were collected by centrifugation at 3000 rpm for 10 minutes in sterilized bottles and resuspended into 1 L of BMMY medium and transferred into 2.8 L flask. The culture was maintained at 30^0 with shaking to start induction of protein. After 24 hours of induction, the cells were removed by centrifugation and the supernatant was frozen at -80^0.

Isolation of inclusion bodies from E.coli

Frozen *E. coli* cell paste was resuspended in cold Milli-Q water at a concentration of 75 mg/ml. The cells were dispersed with a homogenizer for 30min on ice. The cells were then mechanically lysed by sonication. Lysate was centrifuged at 16,000g for 20min. The supernatant was discarded. The inclusion body pellets were collected, resuspended in the same volume of cold Milli-Q water, homogenized, sonicated, and pelleted by centrifugation as above one more time. The inclusion bodies were stored at -80^0.

Sulfonation of inclusion bodies and anion exchange chromatography

The buffers used for sulfonation, anion exchange chromatography, and protein refolding contained high concentration of urea. Urea solutions were treated with Bio Rad mixed bed resin AG®501-X8 at room temperature for at least 20 min and filtered through 0.2 μm filter before mixing with buffers. One gram of inclusion bodies (wet weight) was dispersed in 40ml of a solution containing 50mM Tris/HCl, pH 8.0, and 7.5 M urea by homogenization and vortexing. After the inclusion bodies were largely dissolved, 800mg of sodium sulfide was added, and the mixture was shaken at room temperature for 30min. Then, 400 mg of sodium tetracyanate was added and the mixture was shaken at 4^0 overnight. The solution was dialyzed against 400ml of a solution containing 20mM Tris/HCl, pH 8, and 4 M urea. The dialyzed solution was centrifuged at 48,000xg for 1h, filtered through a 0.2 mm filter, and stored at -80^0. For anion exchange chromatography, 40 ml of sulfonated and dialyzed sample was loaded onto a HiLoad Q-Sepaharose 16/10 column pre-equilibrated in Q-buffer (20 mM Tris/HCl, pH 8-6 M urea-0.01% Brij 35) containing 0.15 M NaCl at room temperature. The column was washed with 240ml of equilibration buffer and then eluted with a 396-ml gradient (0.15-0.4 M NaCl) in Q-buffer at a flow rate of 3 ml/min. Nine ml fractions were collected. The peak fractions containing the wanted protein was analyzed by SDS-PAGE, pooled, and used for refolding.

Refold of disulfide-containing proteins

A standard refold condition developed for refolding of *E. coli*-derived TFPI as described previously (34) was used for refolding of Kunitz inhibitors and ANV:KPI fusion proteins. In brief, the sulfonated and anion exchange chromatography pool was diluted to an absorbance of 0.07 at 280nm with Q-buffer containing 0.3 M NaCl. Solid L-cysteine was added to final concentration of 2 mM. The solution was incubated at room temperature for 24 h, diluted 1:1 with water with addition of 1 mM L-cysteine, and incubated at room temperature for another 24-48 h. For single-domain Kunitz proteins, refold can be carried out at a higher protein concentration (absorbance of 0.15 at 280nm) with essentially the same results.

Purification of 6L15 and ANV-KK$_{TFPI}$

Refold mixture of 6L15 (18ml) was acidified to pH 3.0 by titrating with 1 M citric acid, diluted 1:1 with water, and passed thorough a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 20 mM Na-citrate, pH 3.0. The column was then eluted with a gradient from 0.1 to 1 M NaCl in the same buffer. 6L15 was eluted as a symmetrical peak around 0.5 M NaCl.

Refold mixture of ANV-KK$_{TFPI}$ (600ml) was diluted 1:1 with water and passed through a 1x8cm Q-Sepharose (fast flow) pre-equilibrated with 5 mM Tris, pH 8.0-75 mM NaCl. The column was washed with 50ml of the equilibration buffer. ANV-KK$_{TFPI}$ was then eluted with 5 mM Tris, pH 8.0-0.25 M NaCl.

Purification of TAP-ANV, ANV-6L15, ANV, and ANV-K$_{APP}$.

Refold mixture of TAP-ANV (160ml) was passed though a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 20 mM Tris, 7.4. The column was washed with 50ml of the same buffer containing 0.15 M NaCl, and eluted with a gradient from 0.15 M to 0.35 M NaCl in the same buffer. TAP-ANV was eluted as a single symmetrical peak around 0.33 M NaCl. Refold mixture of ANV-6L15 was loaded on a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 6.7 mM Tris, pH 9.5-2 M urea-0.003 % Brij35-0.1 M NaCl. The column was washed with 40ml of the same buffer, followed by 30ml of 20mM Tris, pH 7.4, then eluted with a 180ml-gradient from 0.1 M to 1 M NaCl in 20 mM Tris, pH 7.4. ANV-6L15 was eluted at around 0.28 M NaCl. The Q-Sepharose purified TAP-ANV and ANV-6L15 were further purified by adsorption to PS-containing liposomes by modification of the method described by Thiagarajan and Benedict (38). Multilamellar liposomes were prepared according to the method of Kinsky (39). Bovine brain extract (100 mg) containing 50% PS, 150mg cholesterol, and 10 mg diacetylphosphosphate were dissolved in chloroform and dried in a stream of nitrogen in a 40-ml glass vial. TBS (10 ml) was added to the vial and agitated vigorously in a vortex mixer for 5 min. The liposome was pelleted by centrifugation at 10,000g for 10min. The Q-Sepharose-purified TAP-ANV or ANV-6L15 was added to the liposome and $CaCl_2$ was added to a final concentration of 5 mM. The mixture was incubated at room temperature for 40 min, and then centrifuged at 10,000g for 10min. The pellet was washed with TBS-5 mM $CaCl_2$ four times by repeating centrifugation and re-suspension cycle as above. TAP-ANV or ANV-

6L15 was eluted from the liposome using a solution containing 10 mM Tris, pH8.0-5 mM EDTA.

Recombinant ANV was directly isolated from *E. coli* lysate by binding to liposomes as described before (38) with some modification. In brief, the *E. coli* pellet was suspended in 50 mM Tris, pH 7.4-10 mM EDTA, and sonicated on ice to obtain lysate. The lysate was stored at -80^0. Aliquot of the lysate was thawed, dialyzed against TBS, and clarified by centrifugation at 15,000g for 30min. The lysate was incubated with liposome in the presence of 5 mM $CaCl_2$ for 40min, followed by washing, centrifugation, and EDTA-elution as described above.

Recombinant ANV-K_{APP} was expressed and secrete into the culture medium of *pichia*. The medium was concentrated about 10 fold, exchanged with a buffer containing 10 mM Tris, pH 7.4-0.15 M NaCl, and clarified by centrifugation at 40,000g for 1h. The medium concentrate was incubated with liposome in the presence of 5 mM $CaCl_2$, followed by washing, centrifugation, and EDTA-elution as described above. All the proteins eluted from liposomes by EDTA solution were subjected to centrifugation at 20,000g for 1h to separate the proteins from majority of the liposomes. To remove residual vesicles, the protein solutions were further filtered through CentriPlus YM-100 (Amicon).

Protein determination

The concentrations of proteins were determined by absorbance at 280nm using theoretical extinction coefficients calculated from amino acid sequence data as described by Gill and von Hippel (40). The following molar extinction coefficients were used: ANV (21,050); TAP-ANV (39,550); ANV-6L15 (28,170); ANV-K_{APP} (31,300); ANV-KK_{TFPI} (30,170); TAP (18,500); 6L15 (7,120); C127- and *E. coli*-derived FL-TFPI (20,650); C127 truncated TFPI (19,370); TFPI1-160 (7,840); X-K1 (14,490).

Amidolytic assays of trypsin and factor Xa inhibitory activities: Determination of stoichiometries of inhibitor-protease interactions

Bovine factor Xa (from American Diagnostica) and porcine trypsin (from Sigma) were titrated with *p*-nitrophenyl *p'*-guandininobenzoate according to Smith (41) and Chase and Shaw (42), respectively, to determine the concentrations of active factor Xa and

trypsin. Inhibitory activities of TAP, TAP-ANV, and ANV-KK_{TFPI} against factor Xa were assayed by amidolysis of S2765. Ten µl of 50 nM bovine factor Xa in DB-buffer (10 mM Tris, pH7.5-0.15 M NaCl-1mg/ml BSA-0.002% Tween 20-0.02 % NaN_3) was mixed with 10 µl inhibitors diluted in the same buffer. After incubation at room temperature for 30 min, 10 µl of the reaction mixture was taken into 96-well plate and mixed with 85 µl of TBS-buffer (50mM Tris, pH 7.5-0.15 M NaCl-0.02% NaN_3) containing 5 mM $CaCl_2$. The absorbance change at 405nm was recorded on SPECTRAmax® PLUS^{384} (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer at room temperature for 60 sec. Stock solution of porcine trypsin was prepared in 50% glycerol-1 mM HCl-20 mM $CaCl_2$ and stored at -20^0. Inhibitory activities of 6L15, ANV-6-L15, and ANV-K_{APP} against trypsin were assayed by amidolysis of S2444. A diluted trypsin solution (23nM) was freshly prepared from the stock in a buffer containing TBS-0.1 mg/ml BSA-20 mM $CaCl_2$. Ten ul of the trypsin solution was mixed with 10 µl of inhibitors diluted in the same buffer in the microplate well. After incubation at room temperature for 10min, 75 µl of TBS-20 mM $CaCl_2$ and 5µl of 10mM S2444 was added to the mixture and the absorbance change at 405nm was recorded on the microplate spectrophotometer at room temperature for 2min. In both assays, the fractional activities in the presence of inhibitors were calculated as percentages of that in the absence of inhibitors.

Plasma clotting time assays

Human plasma clotting assays were carried out on an ACL 200 coagulation analyzer (Instrumentation Laboratory, Lexington MA). A pooled normal plasma from 4 donors was used. For tissue factor-induced plasma clotting assay, each sample contains 100 µl of pooled plasma mixed with equal volume of an inhibitor dissolved in DB-buffer (10 mM Tris, pH 7.4-0.15 M NaCl-1 mg/ml BSA- 0.02% NaN_3) at varying concentrations. Inhibitor concentrations were calculated as nanomolar in plasma alone, not final plasma-buffer mixture. Innovin® (recombinant human tissue factor reconstituted with synthetic phospholipids) was diluted 1:100 with PT-buffer (75 mM NaCl-12.5 mM $CaCl_2$-0.5 mg/ml BSA-0.02 % NaN_3) for the assay. For activated partial thromboplastin time (APTT) assay, each sample contains 180 µl of pooled plasma mixed with 20µl of an inhibitor dissolved in DB-buffer at varying concentrations. Inhibitor concentrations were calculated as the final concentration in the plasma-buffer mixtures. APTT-SP reagent (Instrumentation

Laboratory) was used for the assay without dilution.

Results

Construction and expression of recombinant ANV and ANV:KPI fusions

Plasmid vectors were constructed and used for expression of recombinant ANV and its fusion proteins with various Kunitz-type protease inhibitors possessing specific inhibitory activities against four key coagulation enzymes, factor VIIa, factor IXa, factor Xa, and factor XIa in the clotting cascade. Fig. 2. schematically depicts the molecular structures of these proteins. ANV was expressed as a full-length un-mutated molecule in *E. coli*. For other ANV-KPI fusions, the Cys^{315} of ANV was mutated to Ala to avoid forming disulfide bonds with the cysteines within the Kunitz domains during protein refolding. The fusion protein of TAP-ANV is total of 382 amino acid residues starting with an Ala residue followed by a 60 amino acids of TAP from Tyr^{1} to Ile^{60}, a dipeptide Gly-Ser, and a 319 amino acids of ANV(Cys^{316}-to-Ala). The fusion protein of ANV-6L15 is total of 378 amino acid residues with a 319 amino acids of ANV(Cys^{315}-to-Ala) from initial Ala to the final amino acid (Asp) followed by a 60 amino acids of 6L15 from Met^{1} to Ala^{60}. In order to create the *Nsi*I restriction enzyme site for gene editing and ligation, the second amino acid of 6L15 in the fusion protein was changed from Ala to His. The fusion protein of ANV-K_{APP} is a polypeptide of 376 amino acids in total length. The N-terminus is the full length ANV(Cys^{315}-to-Ala) and the C-terminus is a 57 amino acids of K_{APP} polypeptide from Asp^{1} to Ile^{57}. The fusion protein of ANV-KK_{TFPI} is a polypeptide of 459 amino acid residues in length. The N-terminus of this fusion protein is a full length ANV(Cys^{315}-to-Ala) fused with a 140 amino acid polypeptide starting from Met^{22} to Thr^{161} of TFPI protein, including Kunitz domains 1 and 2.

Purification of ANV and ANV:KPI fusion proteins

Recombinant ANV, TAP-ANV, ANV-6L15, and ANV-KK_{TFPI} were expressed intracellularly in *E. coli*. Essentially all the ANV molecules present in the *E. coli* lysate were capable of binding to PS-containing liposomes in the presence of Ca^{++} when analyzed by SDS-PAGE, suggesting that the expressed protein spontaneously folded itself into active forms. For other *E.coli*-expressed ANV:KPI fusions, majority of the expressed proteins occurred in inclusion bodies and required refolding to obtain active molecules.

Using a sulfonation refold process developed previously for TFPI (34), we were able to achieve refolding of ANV:KPI fusion proteins as evidenced from the increase in inhibitory activity against trypsin or factor Xa during refolding. One-step Q-Sepharose chromatography of a refold mixture achieved high degree of purification as a single major band with the expected apparent molecular mass was observed in SDS-PAGE analysis. Further purification was carried out by binding to PS-containing liposomes in the presence of Ca^{++} followed by elution with EDTA. Recombinant ANV-K_{APP} was expressed and secreted into the culture medium of *P. pastoris* in active form. Active ANV-K_{APP} can be purified from concentrated medium by binding to PS-containing liposome in the presence of Ca^{++} and elution with EDTA. SDS-PAGE analysis of the final purified products is shown in Fig. 3. Under non-reducing condition (Fig. 3A), a major band was seen in each preparation. ANV-KK_{TFPI} (lane 2) and ANV (lane 6) both contained traces of dimmers. Under reducing condition (Figure 3B), the dimers disappeared and the fusion protein bands migrated slightly slower possibly because of disruption of disulfide bonds and unfolding of the Kunitz domains.

Stoichiometries of the interaction of the purified inhibitors with trypsin or factor Xa

Fig. 4 shows the titrations of trypsin or factor Xa activities by the purified inhibitors. Except ANV-KK_{TFPI}, the purified fusion inhibitors (ANV-6L15, TAP-ANV, and ANV-K_{APP}) and the Kunitz inhibitors (6L15 and TAP) all inhibits trypsin or factor Xa with apparent stoichiometries of 1:1. These results indicated that all the purified inhibitors containing a single Kunitz domain were substantially pure and fully active. The extent of deviation from 1;1 stoichiometry observed near equimolar concentration of inhibitor and enzyme reflects the variation in affinity of the interactions. The affinities of ANV-6L15, 6L15, and ANV-K_{APP} for trypsin (Fig 4 A, B, and E) appear stronger and the associations of TAP-ANV and TAP with factor Xa (Fig. 4C, and D) appear weaker. Evidence of weaker affinities of TAP-ANV and TAP with factor Xa are also inferred from time-dependent slow increases of amidolytic activity upon addition of substrate and buffer in the assay. Titration of Xa with ANV-KK_{TFPI} showed diviation from 1:1 stoichiometry (Fig. 4F). This is possibly due to the weak binding affinity of TFPI-K2 for Xa (Ki=90 nM) (43), thus the stoichiometry of interaction cannot be determined under the experimental condition used. Alternatively, the purified ANV-KK_{TFPI} may contain inactive misfolded species.

Prolongation of tissue factor-initiated clotting time

It is well established that TF is the physiologic trigger of blood coagulation. Therefore, the anticoagulant effects of various inhibitors were examined in TF-initiated plasma coagulation assay. Purified inhibitors were added to pooled human plasma at different concentrations and plasma clotting was initiated by adding a diluted thromboplastin reagent (1:100 dilution of Dade Innovin®). Innovin® is a commercial preparation of recombinant human TF reconstituted with an optimized phospholipid mixtures. The assay reagent contains both TF and anionic phospholipid to allow initiation and propagation of the coagulation cascade, and is a simplified system mimicking plasma clotting in the presence of activated TF-bearing cells/microparticles and platelets. The clotting time of the pooled plasma with added control buffer was 40.7 sec. With increasing concentration of added inhibitors, the clotting time was progressively prolonged. The concentration of inhibitors prolonging the clotting time 1.5 fold (i.e. from 40.7 to 61.1 sec) can be determined from the concentration-clotting time curves. Table 1 shows the concentrations required to prolong clotting time 1.5 fold for various inhibitors and their relative potency ranking. Since TFPI is the most important physiological regulator of the tissue factor pathway of coagulation in blood, and mammalian cell-derived TFPI may resemble most the former, we have chosen recombinant C127 FL-TFPI as a reference standard for comparison. TAP-ANV, presumably targeting the prothrombinase, is 86-fold more potent compare to C127 FL-TFPI. ANV-6L15, designed to inhibit TF/VIIa, is 12 fold more potent than C127 FL-TFPI. ANV-K_{APP} (possibly targeting TF/VIIa, XIa, VIIIa/IXa, and Va/Xa), ANV-KK_{TFPI} (presumably inhibiting TF/VIIa and Va/Xa), *E. coli*-derived non-glycosylated TFPI (presumably inhibiting TF/VIIa/Xa), and X-$K1_{TFPI}$ hybrid (likely inhibiting TF/VIIa) are 6-7 fold more potent than C127 FL-TFPI. ANV alone is 2.4 fold more potent than C127 FL-TFPI. TAP has the same potency as C127 FL-TFPI. Kunitz inhibitors alone, as exemplified here by TFPI1-160 and 6L15, are 40- and 59-fold, respectively, less active than C127 FL-TFPI.

Effects of various inhibitors on APTT

The anticoagulant effects of various inhibitors were also examined by activated partial thromboplastin time (APTT) assay. APTT measures the intrinsic pathway activity. The effects of various inhibitors in prolonging APTT are shown in Fig. 5. For the purpose of

comparison, ANV is chosen as a reference standard. The most potent molecule, TAP-ANV, is about an order of magnitude more potent than ANV. The effect is likely mediated through inhibition of prothrombinase. ANV-K_{APP} (presumably inhibiting XIa, VIIIa/IXa, and Va/Xa), ANV-KK_{TFPI} (presumably inhibiting Va/Xa), and ANV-6L15 (possibly inhibiting kallikrein and XIa) are several-fold more potent than ANV. The Kunitz inhibitors alone (6L15, TAP, and TFPI1-160) are very weak in prolonging APTT. Interestingly, glycosylated mammalian C127 FL-TFPI is about an order of magnitude more potent than non-glycosylated *E. coli*-derived TFPI (Fig 5), the order of potency being reversed vs. that of tissue factor-induced clotting (Table 1). These results suggest that there are significant differences between mammalian- and *E. coli*-derived TFPIs.

Table 1:

Effects of various inhibitors on tissue factor-induced clotting time in human plasma.

Inhibitor	[a] $[Inhibitor]_{1.5xCT}$, (nM)	[b] Relative potency
TAP-ANV	0.80	86
ANV-6L15	6.0	12
ANV-K_{APP}	9.4	7.3
X-$K1_{TFPI}$	10	6.9
ANV-$KK_{TFPI22-160}$	11	6.3
E. coli ala-TFPI	19	6.3
ANV	29	2.4
TAP	68	1
C127 FL-TFPI[c]	69	1
C127 CT-TFPI[c]	1300	0.053
E. coli TFPI1-160	2750	0.025
6L15	5900	0.017

[a] $[Inhibitor]_{1.5CT}$ is the concentration of inhibitor that prolong the tissue factor-induced clotting time 1.5 fold relative to control (from 40.7 to 61.1sec) as determined from concentration-dependent clotting time curves for each inhibitor.

[b] Relative potency is calculated from $[Inhibitor]_{1.5CT}$ using mammalian C127 FL-TFPI as reference standard (assigning C127 FL-TFPI as 1).

[c] C127 FL-TFPI refers to full-length molecules; CT-TFPI refers to molecules truncated at the carboxyl terminus as described previously (33).

Although the inventor is not to be bound by theory, it is believed that the foregoing results can be explained and elaborated thereon as follows:

Formation of extrinsic tenase (TF/VIIa), intrinsic tenase (VIIIa/IXa), prothrombinase (Va/Xa) and XIa enzymatic complexes on anionic membrane surfaces are the key processes by which initiation and propagation of the tissue factor pathway of coagulation occur. TFPI is the primary physiological regulator of the initiation of coagulation. TFPI does not directly inhibit TF/VIIa complex per se, but in stead, must await generation of factor Xa first before forming an inert quartnary TFPI/Xa /TF/VIIa complex (17). Generation of factor Xa leads to formation of prothrombinase (Va/Xa), and once formed, prothrombinase is protected from inactivation by physiological concentration of TFPI (44,45). During this process, some intrinsic tenase (VIIIa/IXa) is also generated which is not inhibited by TFPI. As a result, TFPI regulates tissue factor pathway in a rather "leaky" manner. In *in vitro* clotting assay using 1:100 dilution of a commercial thromboplastin reagent, it requires 69 nM of mammalian cell-derived full-length TFPI to prolong clotting time just 1.5 fold (Table 1). In *in vivo* thrombosis models, efficacies were observed only when high concentrations of TFPI (100-200 nM) are present in circulating blood or topically (18,19,46). These therapeutic doses of TFPI represent about 100-200 fold of that in the normal plasma. The apparent low potency and the large infusion dose required to achieve the desired blood level make TFPI less than ideal for therapeutic applications. Hence, it is highly desirable to have alternative molecules that exert better control of the tissue factor pathway of coagulation. The ANV:KPI fusion proteins created herein are far more potent than TFPI in the inhibition of TF-initiated coagulation and possess other advantages over TFPI as therapeutic anticoagulants.

Coagulation cascade reactions are localized on PS-exposed membrane surfaces that facilitate the assembly of the coagulation complexes and enhance the catalytic efficiency. In the present work, it is hypothesized that enzyme inhibitors that have been conferred the ability to target themselves to the PS-exposed membrane surface would become thrombogenic site-specific and more effective in inhibiting the coagulation complexes. To test this hypothesis, recombinant DNA technology is used to create four fusion proteins that share a common ANV domain linking to different KPI domains (TAP, 6L15, K_{APP}, and KK_{TFPI}). The ANV moiety has high affinity (K_d <0.1 nM) for membranes containing PS (32). The four KPIs chosen for this study have the following inhibition constants (K_i) for various coagulation serine proteases: TAP (0.18 nM for Xa) (22); 6L15 (0.2 nM for

TF/VIIa, 0.02 nM for plasma kallikrein and 13 nM for XIa) (30,31); K_{APP} (68 nM for TF_{219}/VIIa; 13 nM for Xa; 190 nM for IXa; and 0.01 nM for XIa.) (24-27); and $K1K2_{TFPI}$ (90 nM for Xa and 240 nM for TF/VIIa) (43). Based on the specificities of these KPIs, the fusion proteins are presumed to preferentially target various membrane-associated coagulation enzyme complexes as follows: TAP-ANV for Va/Xa; 6L15-ANV for TF/VIIa, kallikrein, and XIa; K_{APP} for XIa, Va/Xa, TF/VIIa, and VIIIa/IXa; and ANV-KK_{TFPI} for Va/Xa and TF/VIIa. In *in vitro* clotting assays, the KPIs all require fairly high concentrations in plasma to prolong clotting times (30, 47, Table 1 and Figure 5). All the four ANV:KPI fusion proteins, in contrast, prolong the plasma clotting times at greatly reduced concentrations compared to their component ANV and KPIs (Table 1 and Fig. 5). In both TF-induced plasma clotting and APTT assays, the most potent fusion protein is TAP-ANV. This molecule inhibits prothrombinase since TAP is a highly specific inhibitor of factor Xa. The result is consistent with the finding that factor Xa and prothrombinase generation is the rate-limiting step in the coagulation cascade (3). It is significant to note that 6L15 is a very poor inhibitor of TF-initiated plasma clotting (Table 1) in spite of its high affinity binding with TF/VIIa (K_i 0.2 nM) (30). Thus, high affinity binding of TF/VIIa alone does not correlate with good potency in inhibiting TF-initiated clotting cascade. In contrast, the fusion inhibitor of ANV-6L15 is about three-order of magnitude more potent than 6L15 in inhibiting TF-initiated clotting, indicating that binding to PS greatly facilitates the inhibition of TF/VIIa by 6L15. The four ANV:KPI fusion molecules created herein all show much higher anticoagulant activities than ANV, KPIs, and TFPI as assessed by TF-initiated plasma clotting and APTT assays. Thus. these molecules possess superior anticoagulant potencies to those of the natural anticoagulants.

In vivo animal study has demonstrated that ANV can specifically target and accumulate on platelet-containing thrombi (48). Furthermore, ANV preferentially accumulates at sites of vessel injury and dose-dependently inhibits thrombus formation in arterial and venous thrombosis models (38,49,50). An important attribute of the ANV:KPI fusion proteins described herein is the presence of ANV moiety that confers on them the property of binding specifically to PS with high affinity. Thus, these molecules possess an intrinsic property of targeting themselves to sites of thrombus formation where PS becomes available for assembly of coagulation complexes. Owing to their ability to target thrombogenic sites, it will be feasible to achieve antithrombotic effect without maintaining high levels of these anticoagulants in systemic circulation, thereby minimizing risks of

systemic bleeding side effect.

As used herein, the term "pharmaceutically acceptable" refers to a characteristic of carriers, excipients, or other additives commonly used to formulate drug compositions for administration to subjects for the treatment of diseases and conditions.

As used herein the term "subject" refers to both a human or an animal having or suspected of an excess of thrombotic actvity.

The terms "effective" and "therapeutically effective" as used herein refer to a characteristic of an amount of a therapeutic compound or composition, wherein when administered to a subject, the amount achieves one or more of the goals of preventing, inhibiting, reducing or eliminating a disease or condition being treated in the subject. With respect to the present application, diseases and conditions amenable to treatment according to the methods and materials provided, include any disease or condition involving an excess of thrombogenesis. Such diseases and conditions include, for example, unstable angina, myocardial infarction, sudden cardiac death, ischemic stroke, ruptured aneurisms, intermittent claudication, critical limb ischemia; deep venous thrombosis, pulmonary embolism, thrombophlebitis, chronic venous insufficiency; surgical thrombosis, prosthetic heart valve, atherosclerosis, restenosis, ischemia reperfusion injury, sepsis, disseminated intravascular coagulation, acute lung injury, malignancy, chronic renal failure, nephrotic syndrome, crescentic glomerulonephritis, diabetes, sickle cell disease, thalassemia, antiphospholipid syndrome, extra-corporeal circulation, hemodialysis, peritoneal dialysis, and annexinopathies.

The terms "dosing" and "treatment" as used herein refer to any process, action, application, therapy or the like, wherein a subject, particularly a human being, is rendered medical aid with the object of improving the subject's condition, either directly or indirectly.

The term "therapeutic compound" as used herein refers to a compound useful in the prophylaxis or treatment of a thrombogenesis-related disease or condition.

As demonstrated herein, this series of recombinant proteins can be produced in *E. coli* and yeast. In the *E. coli* system, the proteins can be expressed at very high levels in the inclusion bodies, and active molecules can be obtained by simple refold and purification procedures. In the *Pichia* system, the protein can be secreted into the culture medium in active form and purified by the same simple procedure. From a manufacturing

standpoint, ease and low cost of production are of great advantage. However, it is contemplated that other prokaryotic and eukaryotic cell lines can be used as according to well known general molecular biology procedures as described in, for example, in Sambrook, et al., MOLECULAR CLONING--A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory, 1989).

Based on the results of the present work, it is believed that other fusion molecules of similar conceptual design can be created. For example: fusion proteins consisting of multiple ANV domains or KPI domains; fusions of ANV with other natural inhibitors of coagulation factors, such as Antistasin, ecotin (51), Acylostoma caninum anticoagulant peptides; fusions of ANV with homologs and variants of KPIs; and fusions of ANV with small-molecule inhibitors of factors VIIa, IXa, Xa, and XIa. In another variation, other PS binding proteins such as other members of annexin family, lacadherin (52), and phospholipid binding moieties of factor V, factor VIII and phospholipase A_2 can be used in place of ANV for the creation of fusion molecules. In a further example, disintegrin domains can be linked to ANV or ANV-KPIs to create fusion molecules that inhibit both coagulation reactions and platelet aggregation at sites of thrombogenesis.

All such other examples as will be apparent to the person skilled in the art after reading the present disclosure are intended to be included within the scope of the present invention.

In summary, the newly developed ANV:KPI fusion proteins represent a new class of thrombogenic site-targeted anticoagulants with the following characteristics: (a) They are designed to target TF/VIIa, intrinsic tenase (VIIIa/IXa), prothrombinase (Va/Xa) and XIa associated with PS-exposed thrombogenic membranes; (b) They possess 6-86 fold higher anticoagulant potencies than TFPI, the natural inhibitor of coagulation initiation, in TF-initiated coagulation; (c) Ease and low cost of production, because fully active molecules can be produced by microbial systems and purified by simple Ca^{++}-dependent binding to PS-containing liposome followed by elution with a Ca^{++}-chelating solution; and (d) Because PS-exposed membranes and associated coagulation complexes are key thrombogenic stimuli at vascular lesions, ANV:KPIs are potentially useful antithrombotic drugs capable of localized passivation of thrombogenic vessel walls and associated thrombi.

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